

# Severe outcome of influenza A/H1N1/09v infection associated with 222G/N polymorphisms in the haemagglutinin: a multicentre study

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## Abstract

In a multicentre study, influenza A/H1N1/09v 222G/N variants were more frequently detected in patients admitted to the intensive-care unit for invasive mechanical ventilation or extracorporeal membrane oxygenation (10/23; 43.5%) than in patients hospitalized in other units (2/27; 7.4%) and community patients (0/81; 0.0%) ( $p < 0.01$ ). A significantly higher virus load ( $p = 0.02$ ) in the lower vs the upper respiratory tract was observed. Predominance of 222G/N variants in the lower respiratory tract (40% of total virus population) vs the upper respiratory tract (10%) was shown by clonal analysis of haemagglutinin sequences in paired nasal swab and bronchoalveolar lavage samples. The time from illness onset to sampling was significantly longer in patients with severe infection vs community patients ( $p < 0.001$ ). It was concluded that the 222G/N variants showed increased virulence; mutant variants were probably selected in individual patients; and the longer duration of illness might have favoured the emergence of adaptive mutations through multiple replication cycles.

**Keywords:** 222G/N variants, bronchoalveolar lavage, intensive-care unit, influenza A/H1N1/09v, virulence

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## Introduction

The 222G/N polymorphisms in the haemagglutinin (HA) gene of influenza A/H1N1/09v pandemic virus have been associated with cases of mild to severe illness from different countries or geographical areas [1]. Mutant viruses have been reported since April 2009, but no distinct phylogenetic clusterings or consistent changes in virus antigenicity have been observed [1]. Hence, it was suggested that the mutations occurred sporadically, as opposed to the sustained transmission of a variant virus. Recent studies from Norway, Hong Kong, Scotland and Spain have shown a higher prevalence of 222G/N in patients with severe illness [2–5], whereas the polymorphism 222E was not associated with increased virulence [2]. In addition, the low prevalence of the 222G variants in fatal cases

(26/364, 7.1%) and the limited information about potential underlying medical conditions in these cases make it difficult to determine the clinical relevance of this polymorphism [1]. Patients in these studies were stratified on the basis of broad definitions of the severity of the influenza-like illness (ILI) in the absence of precise clinical parameters. The clinical impact of these amino acid polymorphisms is still debated [2].

In this report, the association of polymorphisms at codon 222 of the virus HA with the severity of the clinical presentation was investigated in a multicentre study in three groups of patients with influenza A/H1N1/09v infection.

## Materials and Methods

Two reference centres for surveillance of pandemic influenza A infection in Pavia and Milan as well as two reference intensive care units (ICU) for advanced critical care including extracorporeal membrane oxygenation (ECMO) procedure, in Pavia and Palermo, participated in the study. Following the criteria indicated by Zarychanski *et al.* [6], patients were

stratified as follows: (i) group A (severe ILI) consisted of 23 sequential patients (males 13/23, 56.5%, median age 35 years, range 11–60 years) with acute respiratory distress syndrome characterized by bilateral alveolar infiltrates on chest radiography and rapidly progressive hypoxaemia [7], admitted to ICUs for invasive mechanical ventilation or ECMO procedures; (ii) group B (moderate ILI) consisted of a comparable number ( $n = 27$ ) of sequential patients (males 17/27, 62.6%; median age 43 years, range 1–85 years) with lower respiratory tract infection, (positive chest radiography and/or reduced  $pO_2$  blood saturation,  $pO_2 < 90\%$ ), requiring admission to units other than ICUs for antiviral and/or oxygen supplementation by non-invasive assisted ventilation procedures; (iii) group C comprised a three-fold greater number of patients ( $n = 81$ ), consisting of patients (males 43/81, 53.0%; median age 21 years, range 3–77 years) with mild ILI (control group). The 81 controls were randomly selected among patients diagnosed with influenza A/H1N1/09v infection at the reference centres in Pavia and Milan during the national surveillance programme in the period April–November 2009, and consisted of patients with community-acquired self-resolving ILI, not requiring hospitalization, administration of antiviral drugs or oxygen supplementation (mild ILI).

Clinical decisions were based upon prospective diagnosis of influenza A/H1N1/09v performed by a real-time RT-PCR (Centers for Disease Control, Atlanta, GA, USA), whereas patient stratification, virus load determination and HA gene analysis were performed retrospectively. In detail, virus load in nasal swab and bronchoalveolar lavage (BAL) samples was obtained by interpolation of real-time RT-PCR threshold values from clinical samples on a standard curve obtained by parallel amplification of quantitative standards consisting of serial dilutions of a recombinant plasmid construct carrying the HA target sequence. The presence of amino acid polymorphisms at codon 222 of the HA gene of influenza A/H1N1/09v strains was determined by RT-PCR amplification of the HA gene directly from clinical specimens. An amplicon of 800 base pairs was obtained using H1N1v-specific primers (HA-swF2, GCA AGC TCA TGG TCC TAC ATT GTG GA; HA-swR3, CCC TTC AAT GAA ACC GGC AAT GG). The RT-PCR were carried out using the Ag-Path-ID one-step RT-PCR kit (Applied Biosystems, Foster City, CA, USA) in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) using the following thermal profiles: one cycle at 50°C for 15 min and 95°C for 10 min, followed by 50 cycles at 95°C for 60 s, 60°C for 60 s and 72°C for 70 s, with a final elongation of 7 min at 72°C. Sequencing of the HA amplicon was performed with internal primers (HA-swF3, TAA CGG CAG CAT GTC CTC ATG CTG GA; HA-swR2,

GAG GCT GGT GTT TAT AGC ACC CTT GG) using the BigDye Terminator Cycle-Sequencing Ready Reaction and ABI Prism 3100 DNA sequencer (Applied Biosystems). Sequences were then assembled and aligned using SEQUENCHER (version 4.7; Gene Code Corp., Ann Arbor, MI, USA) and BIOEDIT software.

The relative proportion of wild-type and mutant viruses in the upper vs lower respiratory tract was analysed by clonal analysis of HA sequences from paired nasal swab and BAL specimens. Briefly, HA amplicons were cloned into the pCR2.1 plasmid vector (TA Cloning kit; Invitrogen, Groningen, the Netherlands) and ten plasmid clones from each specimen were individually sequenced following in principle a previously reported procedure [8].

Comparison of viral load was performed with the Mann–Whitney rank-sum test for continuous variables and the chi-square test or Fisher's exact test for categorical variables was used for analysis of 222G/N prevalence.

## Results

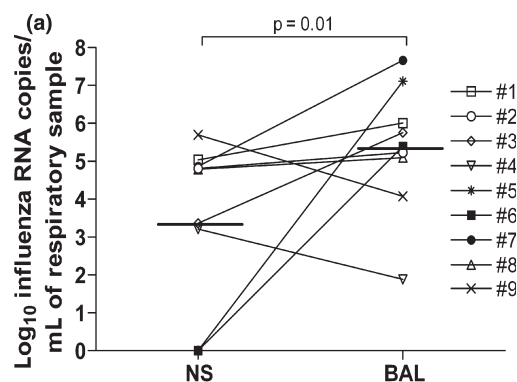
Virus strains with the 222G/N polymorphisms were more frequently detected in patients of group A (10/23, 43.5%) with respect to both groups B (2/27, 7.4%) and C (0/81, 0.0%) ( $p < 0.01$ ). A similar trend was observed also between groups B and C ( $p 0.06$ ).

The median virus load in nasal swabs of patients in groups A ( $5.7 \times 10^4$ , range  $1.0 \times 10^0$  to  $6.9 \times 10^6$ ) and B ( $6.5 \times 10^4$ , range  $1.0 \times 10^0$  to  $1.7 \times 10^8$ ) was significantly lower than that detected among patients in the control group ( $3.1 \times 10^6$ , range  $1.0 \times 10^0$  to  $9.0 \times 10^8$ ) ( $p 0.01$ ).

The time span from illness onset to sampling of patients was significantly longer in patients of groups A (4.5 days, range 1–6) and B (5.5 days, range 1–10) with respect to group C (1 day, range 1–6) ( $p < 0.001$ ). On the other hand, the time span from illness onset to sampling of patients of groups A and B with the 222G/N variants (6 days, range 4–7) compared with the patients of groups A and B with the 222D/E variants (3 days, range 1–10) was not significantly different ( $p > 0.05$ ).

In nine patients (seven in group A and two in group B) paired nasal swab and BAL samples were available. In these patients, virus load was significantly higher in the lower respiratory tract (median  $2.4 \times 10^5$  RNA copies/mL) than in the upper respiratory tract (median  $2.8 \times 10^3$  RNA copies/mL) ( $p 0.01$ ) (Fig. 1a).

In two of these patients (pt#5 and #6), the HA sequence from the nasal swab could not be obtained because of low virus load ( $< 10$  virus RNA copies/mL). In four patients



(b)

Patient No.	Sample date	ILI	NS	BAL
1	27/Oct/2009	severe	222E	222E
2	05/Nov/2009	severe	222D/N	222D/G/N
3	09/Nov/2009	severe	222D	222D/G/N
4	25/Nov/2009	severe	222E/D	222E/D
5	6/Nov/2009	severe	ND <sup>a</sup>	222D
6	21/Nov/2009	moderate	ND <sup>a</sup>	222D/G
7	5/Nov/2009	moderate	222D	222D
8	18/Nov/2009	severe	222D	222D/G
9	18/Nov/2009	severe	222E	222E/G

<sup>a</sup>ND, not done because of low virus load (<10 virus RNA copies/mL)

**FIG. 1.** (a) Influenza A/H1N1/09v load in paired nasal swabs (NS) and bronchoalveolar lavage (BAL) samples of seven patients with severe influenza-like illness (ILI) and two patients with moderate ILI. (b) Influenza A/H1N1/09v amino acid polymorphisms (predominance) in paired NS and BAL samples of seven patients with severe ILI and two patients with moderate ILI.

(44.4%), no 222G/N variants were detected in either nasal swab or BAL, but the remaining five patients (55.5%) showed the presence of 222G/N variants. In three out of four (75.0%) patients with 222G/N variants and available HA sequence in both nasal swab and BAL, the mutant viruses were detected only in the lower respiratory tract, whereas in a single patient (pt#2) a 222N variant was detected both in nasal swab and BAL samples (Fig. 1b). In these four patients (pt#2, #3, #8 and #9), the relative proportion of wild-type and mutant viruses in the upper vs lower respiratory tract was investigated by clonal analysis of HA sequences. For comparison, ten HA plasmid clones were also obtained from the nasal swab and BAL samples of pt#4, who did not show the presence of 222G/N variants in either specimen (Fig. 1b). The 222G/N variants were detected at clonal level in all patients. However, a significantly ( $p < 0.001$ ) higher proportion of mutant variants was observed in the lower (median 40% clones, range 20–90%) than the upper (median 10% clones, range 0–60%) respiratory tract. Interestingly, in pt#2, who showed by direct sequencing 222G/N variants in both nasal swab and BAL, a comparable number of mutant variant specimens was observed by clonal analysis

in both specimens (six of ten clones, 60% and five of ten clones, 50%, respectively). Although direct sequencing of HA amplicons from nasal swabs of pt#3, #8 and #9 did not detect mutant viruses, a minor proportion of 222G/N variants was shown in pt#3 and #9 by clonal analysis (one of ten clones, 10%). Similarly, a minor proportion of 222G/N variants not detected by direct sequencing was shown in nasal swab (one of ten, 10%) and BAL (two of ten, 20%) clones of pt#4.

## Discussion

Using a stringent clinical definition of the severity of ILI in a multicentre prospective study, the association between the emergence of influenza A/H1N1/09v 222G/N virus variants and increased virulence was documented. Frequency of 222G/N variants in patients with severe ILI was strikingly higher than that in patients with moderate or mild ILI. Similarly, patients with moderate ILI more frequently harboured mutant viruses. In addition, it was shown that mutant virus variants segregated preferentially in the lower respiratory tract, where increased virus replication and higher virus load were also shown. Although paired upper and lower respiratory tract specimens at specified intervals from all patients with lower respiratory tract disease could not be systematically obtained in this observational study, the findings here reported support the hypothesized higher affinity of the 222G/N variants for the avian-like  $\alpha 2,3$ -sialic acid receptors present in the low respiratory tract as opposed to the higher affinity of the 222D/E variants for the human-like  $\alpha 2,6$ -sialic acid receptors exclusively present in the upper respiratory tract [9,10]. The observed predominance of 222G/N strains in the lower respiratory tract further strengthens the hypothesis of their fitness in this biological environment. In addition, the frequent detection of mixtures of different virus variants supports the hypothesized sporadic selection of the 222G/N variants in individual patients, as opposed to their circulation in the population. This hypothesis is also supported by the longer duration of illness in patients of groups A and B with respect to group C, which might have favoured the emergence of adaptive mutations in the HA through multiple replication cycles. Indeed, a potential epidemic cluster of 222G/N virus variants in our patients was excluded on the basis of differential nucleotide signatures along the entire HA gene as well as on the unlikelihood of the simultaneous occurrence of such an event in three centres located in northern and southern Italy. On the other hand, the patient clonal analysis showed a small proportion of 222G/N variants not detected by direct sequencing, suggesting that the prevalence of such

variants in patients with severe or moderate ILI might have been underestimated by the routine sequencing assay.

The infection of the lower respiratory tract was more frequently associated with higher virus load with respect to the upper respiratory tract, concomitantly with severe tissue damage and highly impaired O<sub>2</sub> exchange. The prolonged time of illness in patients of groups A and B with respect to patients of group C appears to be associated with the localization of infection from the upper to the lower respiratory tract and, finally with selection of mutant virus variants that fit better into the new environment. In contrast, a reduced ability of mutated virus variants to spread through inter-human infection of the upper respiratory tract might have accounted for the reduced circulation of 222G/N variants and the mild clinical impact of the 2009 influenza A pandemic [11].

In conclusion, the 222G/N polymorphisms in the influenza A/H1N1/09v HA are associated with significant virulence and the virulent variants appear to have arisen sporadically, remaining restricted to the lower respiratory tract of patients with highly impaired respiratory function. The reported low prevalence of 222G/N variants observed during the 2009 pandemic suggests a low circulation of the virulent virus variants, but it also reveals a potential diagnosis bias because of the widespread use of the upper respiratory tract secretions as a specimen of choice also in patients with severe lung infection. Finally, the potential increase in circulation of such variants in the next seasonal epidemics or a reassortment of these viruses with other human or animal influenza A strains poses serious clinical and public health concerns.

Influenza A/H1N1/09v variants with 222G/N polymorphisms showed increased clinical virulence, and detection of such mutants in the next epidemics is mandatory for better management of ILI in individual patients as well as for surveillance purposes.

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## Transparency Declaration

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